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(DETECTION OF (BNRF1) GENE OF EBV IN HODGKIN'S LYMPHOMA IRAQI PATIENT BY REAL TIME PCR)

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ABSTRACT

Background: Hodgkin's lymphoma is type of lymphoid malignancy, it's an unusual cancer because the neoplasm cells constitute only a minority of the total tumor mass, and the cause of developing disease might be attributed to the infectious agent such as virus especially EBV which contributes to the development of Hodgkin's disease in some cases. Recently the pathogenesis of Hodgkin's disease is studied by new technologies applications like conventional PCR and gene expression array analysis. EBV may be play direct or indirect role in the pathogenesis of disease which due to the trigger some mechanism(s) or May due to the presence of an inherited or acquired depression of immunoregulation that effect on the development of disease. **Method:** The study was aimed to detect the BNRF1 gene of EBV in Hodgkin's lymphoma Iraqi patients. The study include 75 Iraqi patients with Hodgkin's lymphoma (25 samples as blood and 50 samples as formalin fixed paraffin embedded (FFPE) tissue blocks) and 10 samples as reactive hyperplasia blocks for control. The blood samples were collected from hematology unit in Baghdad hospital in medical city but paraffin blocks tissue were collected from histopathological laboratories in different hospitals during the period from September 2013 till March 2014. The DNA was extracted from all samples by using specific

kit and used in the experiments of this study. The work was carried out in the molecular oncology unit lab of GSTS pathology Guy's and St. Thomas' NHS foundation Trust Hospital /London/United Kingdom. The DNA which was extracted before was used to detect the existence of BNRF1 gene specific for EBV loaded in patient samples by using real time quantitation PCR (qRT-PCR) with specific primer and probe. **Results:** The EBV DNA genome encoding the non- glycosylated membrane protein (BNRF1) was detected in 40(53.33%) positive cases of 75 Hodgkin's lymphoma samples and no cases positive for EBV in reactive hyperplasia with high significant ($P<0.01^{**}$) . The majority of positive patients Were males 33(82.5%) than females. In addition to the EBV was more frequently detected in mixed cellularity subtype of HL 27(67.5%). **Conclusion:** The QC- PCR assay allows accurate quantification of EBV load and show as a tool to assist in diagnosis and management of EBV related lymphoma patient and cancer related with EBV DNA that may be considered as a tumor biomarker.

KEYWARDS: EBV, Hodgkin lymphoma Iraqi patients, RT-PCR.

INTRODUCTION

Hodgkin lymphoma (HL) also called Hodgkin's disease is a neoplasm that arises from germinal center or post germinal center B cells. HL has a unique cellular composition, containing a minority of neoplastic cells (Reed-Sternberg cells and their variants) in an inflammatory background. It is separated from the other B cell lymphomas based on its unique clinicopathologic features.[1] Hodgkin's lymphoma (HL) which accounts for approximately 15% of all malignant lymphomas is composed of two different disease entities: the rare lymphocyte-predominant Hodgkin's lymphoma (LPHL), making up approximately 5% of cases and the more frequent classical HL representing approximately 95% of all HLs.[2] Several viruses have been shown to play a role in the development of HL like Epstein –Barr virus (EBV), Human T-lymphotropic virus (HTLV) and Human immunodeficiency virus (HIV).[3] Epstein –Barr virus or human herpesvirus4 (HHV4) belongs to the genus lymphocryptovirus within the subfamily of gamma herpes virus. Common features of these viruses are their lymphotropism, their ability to establish latent infection of their host cells and to induce proliferation of latently infected cells; approximately 90 to 95 percent of adults are EBV – seropositive.[4]

MATERIAL AND METHODS

The subjects included in this study were represented as formalin-fixed, paraffin-embedded (FFPE) lymph nodes biopsy tissue blocks (50 cases) that were obtained from patients who had undergone surgical operation done for them, and (25cases) of blood obtain from hematology unit of Baghdad hospital in medical city in Baghdad but paraffin blocks tissue were collected from histopathological laboratories in different hospitals during the period from September 2013 till March 2014. The DNA was extracted from all samples by using QIAamp DNA Mini Kit (Qiagen/Germany) and used in the experiment of this study. The work was carried out in the molecular oncology unit lab of GSTS pathology Guy's and St. Thomas' NHS foundation Trust Hospital /London/United Kingdom.

The Epstein Barr virus was detected quantitation in the samples by used genesig Kit. The Primer Design™ genesig Kit for Human Herpes Virus 4 EBV Genomes is designed for the in vitro quantification of EBV genomes. The target sequence within the BNRF1 gene has previously been shown to be a good genetic marker for EBV in other clinical real time PCR based studies.[5] The kit is designed to have the broadest detection profile possible whilst remaining specific to the EBV genome. The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis. The kit contents were summarized in the table (1).

Table (1): The Primer Design™ genesig Kit contents

Kit contents	Quantity
EBV specific primer/ probe mix(FAM labeled BHQ quenched)	Lyophilized design for 150 reactions
Positive control template	Lyophilized
RNase/DNase – free water	1 vial

The master mix used OasigTM Lyophilized 2xqPCRMastermixkit (not supplied with kit) contents listed in the table (2).

Table (2): The oasigTM lyophilized 2X qPCR Mastermix contents

Component	Quantity
Lyophilized Master mix	3 ampule (50 reactions per glass ampule)
Re-suspension buffer	4 ampule
ROX dye (which can be added as require when the Master Mix is to be used on hardware platforms that use ROX as a passive reference dye)	1 ampule

Preparation of the kits contents

1. Each tube was Pulse- spin in a centrifuge before opening to ensure lyophilized primer and probe mix is in the base of the tube and is not spilt upon opening the tube.
2. The EBV primer/ probe mix were dissolved by RNase/ DNase free water which supplied with kit.
3. The positive control template was dissolved by RNase/ DNase free water.
4. Each glass ampule lyophilized master mix was re-suspended by re-suspension buffer. **(Note Do not replace the re-suspension buffer with water or any other buffer.)** The master mix was then ready to use as a 2X qPCR master mix.
5. Each tube was Vortex thoroughly to ensure complete re-suspension.

Preparation of stander curve dilution series.

The standard curve is generated by performing 5 serial dilutions for target gene (BNRF1) and control gene (MCR1) each dilution in duplicate, to detect differences in their both amplification efficiencies. For maximum accuracy, the serial dilution included the following steps:

The copy number of EBV in serial dilution(2,3,4,5,6) according to the kit was ($2*10^4$ copy/µl; $2*10^3$ copy/µl ; $2*10^2$ copy/µl ; 20 copy/µl ; 2 copy/µl) respectively.

Serial dilutions were then made examined by qRT-PCR to prepare the standard curve for these assays. Then a standard curve is generated by plotting the Ct values against the logarithm of the initial copy numbers. Threshold cycle (Ct) value was calculated for each sample by determining the point at which the fluorescence exceeded a threshold limit of 50. Each run contained a negative control (no template or EBV negative DNA), a positive control (a known amount of EBV copies).

Plate Setup and real time PCR Setup

Real time assay was performed in a 20µl reaction volume containing 10µl of master mix (The oasig^{TM}2x qPCRMastermix), 1µl (EBV primer/probe Mix (FAM labeled, BHQ quenched)), 4µl of RNase/ DNase free water and 5µl of DNA template (samples). After that pipete 15 µl of EBV detection mix into each well according to the real time PCR plate sheet to setup. And 5µl of DNA template was added into each well according to plate setup.

The plate contains positive and negative control and sample for test each sample with duplicate for results accuracy.

The qRT-PCR reaction condition as follow: stage 1: 15min at 37°C, then stage 2: (Enzyme activation) 95°C for 10 min and in a stage 3: Two step cycles achieved (denaturation 95 °C for 10 Sec. and annealing/extending 60 ºC for 1 min) repeated for 50 cycles.

While the SYBR Green assay was performed in a 20 µl reaction volume containing 10µl of master mix (SYBR® Master Mix, Applied Bio system), 0.7µl of primers mix, 4.3µl of RNase free water and 5µl of DNA template. Plate is setup according to the real time PCR plate sheet.

The qRT-PCR reaction parameters for SYBR Green assay where: stage 1: 2min at 50°C, then stage 2: 95°C for 10 min and in a stage 3: two step cycles achieved (denaturation 95 °C for 15 sec. and annealing 60 ºC for 1 min) repeated for 6 cycles: stage 4: two step cycles (denaturation 95 °C for 15 sec. and annealing 61 °C min. for 1 min) repeated for 40 cycles. After the program finished the data were saved and analysis. The two most commonly used methods to analyze data from real-time, quantitative PCR experiments are absolute quantification and relative quantification.[6] Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve depend on logarithmic input amounts of target gene and reference gene relative copy numbers for each individual sample.

The log input amount was calculated from the equations of standard curve that found in the results in figure (2) (3) as the following formula.

Log input amount= (Ct value) – (b)/m

b = y-intercept of standard curve line

m = slope of standard curve line

Then calculate the input amount (copy number) = 10^ (log input amount)

The level of gene expression = Copy Number (target gene)/ Copy Number (gene control)

But Relative quantification relates the PCR signal of the target gene and reference gene. The 2(-Delta Delta C (T)) method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments.

ΔΔCt= ΔCt target – ΔCt reference gene.[7]

RESULTS AND DISCUSSION

The investigation on EBV DNA genome encoding the non- glycosylated membrane protein BNRF1 was detected successfully by using new molecular technique which is Real time PCR with used specific primer and probe. The amplification accuracy of BNRF1 and MCR1 product was noticed by very symmetric or identical threshold cycle (Cts) for the duplicate reactions for both genes (figure 1).

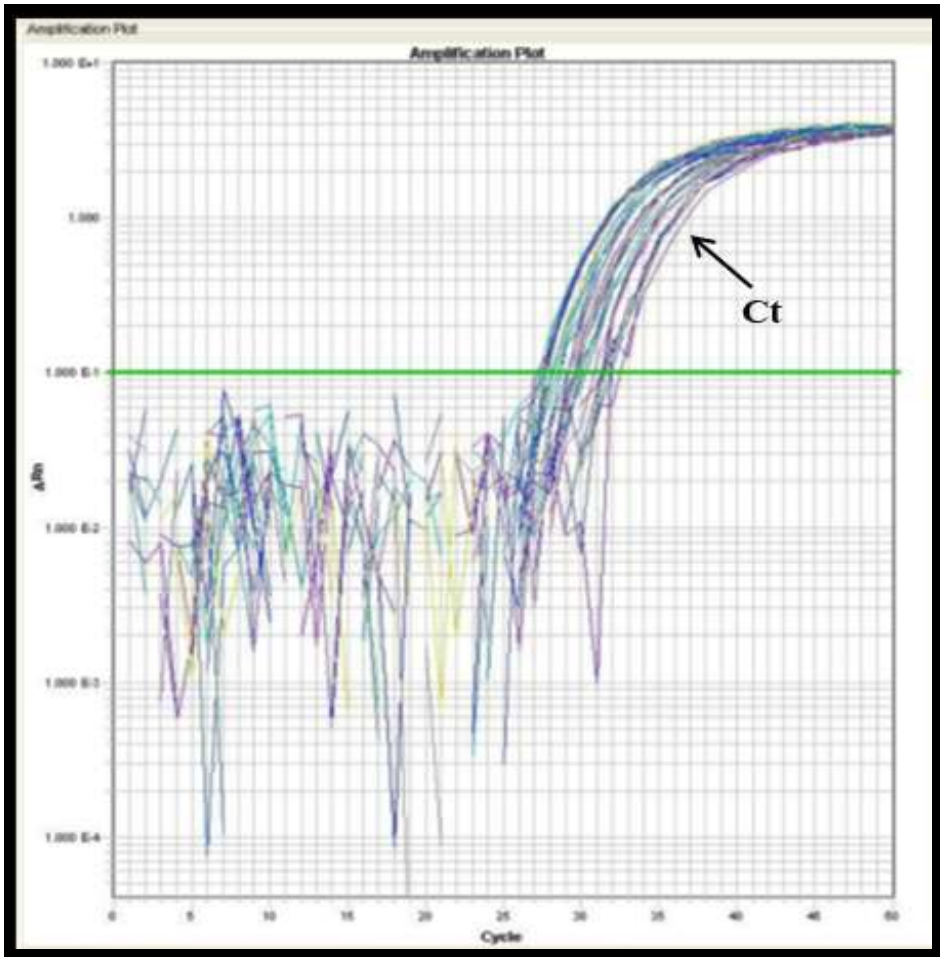

Figure (1): Amplification plot for both the target and reference genes (the threshold fluorescence level as green line)

The results or data obtained from real time experiments were detected according to the Ct values which calculated from cycles 1-15 and was proportional to the starting target copy number (logarithmic scale) used for amplification (the point that the fluorescence signal increased above baseline is the threshold cycle (Ct)) which are inversely related to the amount of starting template that mean the high value of Ct refers to the low levels of gene expression or amplification gene, while low Ct value indicate high level of gene expression or high copy of gene amplification. Amplification plots were appeared when the fluorescent

signal from sample is plotted against cycle number; however amplification plots include the accumulation of product through the period of qPCR experiment.[8] The two common methods used to analyze data from real time PCR experiments are relative quantification and absolute quantification. Absolute quantification detect the copy number in the amplification target, this is often accomplished the PCR signal to stander curve generated using known genome equivalents or nucleic acid harvested from a tittered target control.[9] This method most common used to determine the viral titer because the viral titer determination assay can be complex to design and researchers want to quantify viral copy number in samples.[10]

To determine the dynamic range of real- time PCR amplification, serial dilutions of positive EBV DNA from kit and reference DNA genes were made and subjected to analysis by the real-time quantitative PCR system to prepare standered curve. Figure (2) (3) show the data generated from serial dilution have strong correlation between DNA input a mount and Ct for target and reference genes ($R^2 = 0.99$; $R^2 = 0.099$) respectively. The linearity of the graph (the highest to the lowest quantifiable copy number) demonstrates the large dynamic range and the accuracy of real- time PCR.[11]

According to the suitable dilution for positive control and stander control all Hodgkin's lymphoma cases and control cases were subjected to analysis and detect the EBV positive cases by qPCR amplification. The system was sensitive to detect two copies of EBV DNA in DNA samples.

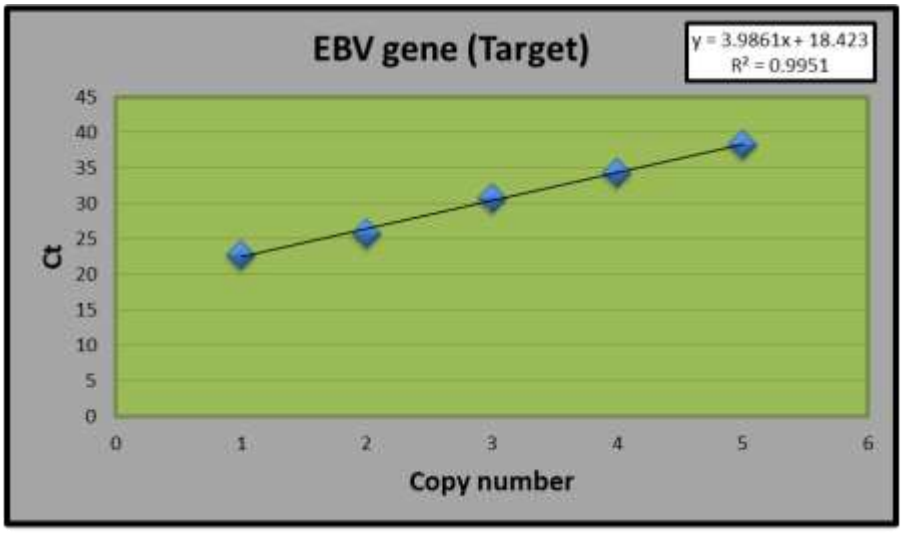

Figure (2): The Curve for serial dilution of BNRF1 gene (the copy number on X- axis and the threshold cycles (Ct) on Y-axis.

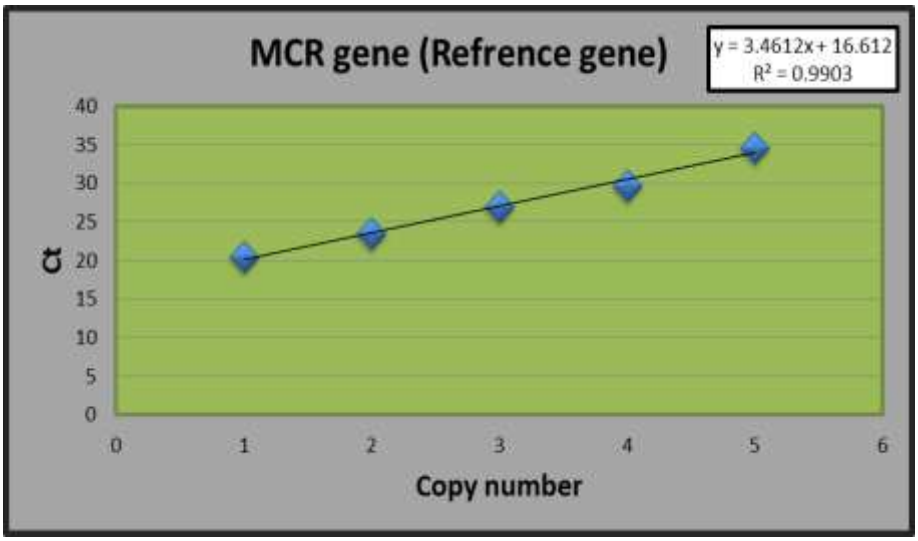

Figure (3): The Curve for serial dilution of reference gene.

The efficiency amplification (E) for both genes (target gene and reference gene) showed high amplification efficiency (97%, 97%) respectively.

By using the equation: $E\% = 10^{(-1/\text{slope of the standard curve})} -1*100$.[12]

The BNRF1 detected in 40(53.33%) of the 75 Hodgkin cases and none of the 10 healthy control with the mean level of 0 copies /µl ($P< 0.001$) (figure 4).

The mean level of EBV DNA in Hodgkin's lymphoma patients was significantly higher than healthy controls at $p <0.001$(Table 4). The median concentration of EBV DNA in Hodgkin's patients groups was 516.00 copies /µl whereas in healthy controls group was 0.0 copies/µl (figure 5).

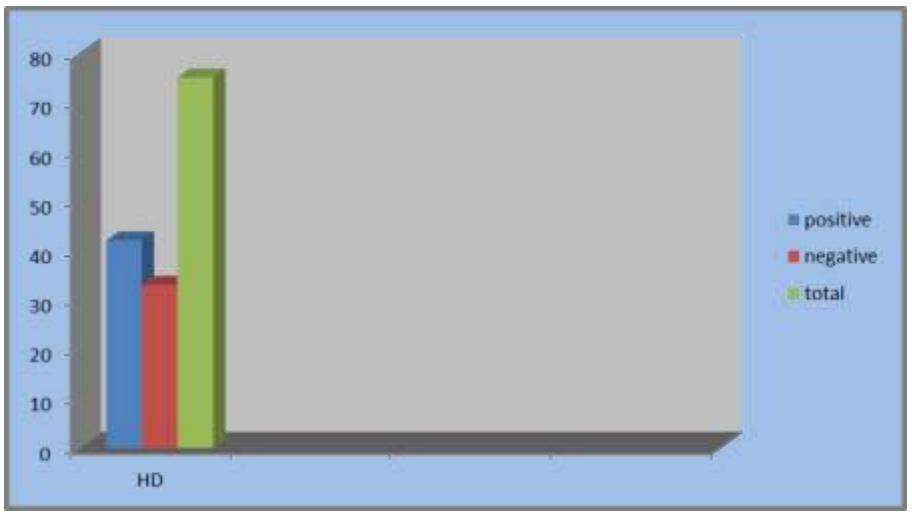

Figure (4): the ratio of positive and negative for EBV DNA detect assay

Table (4-11): The positive cases for EBV assay

Type of samples	Positive	Total
Blood	6(24%)	25
Tissue	34(68%)	50
Control	0.00	10
Total	40(53%)	75patient+10 control
Chi-square value	10.772 **	----
** (P<0.01).		

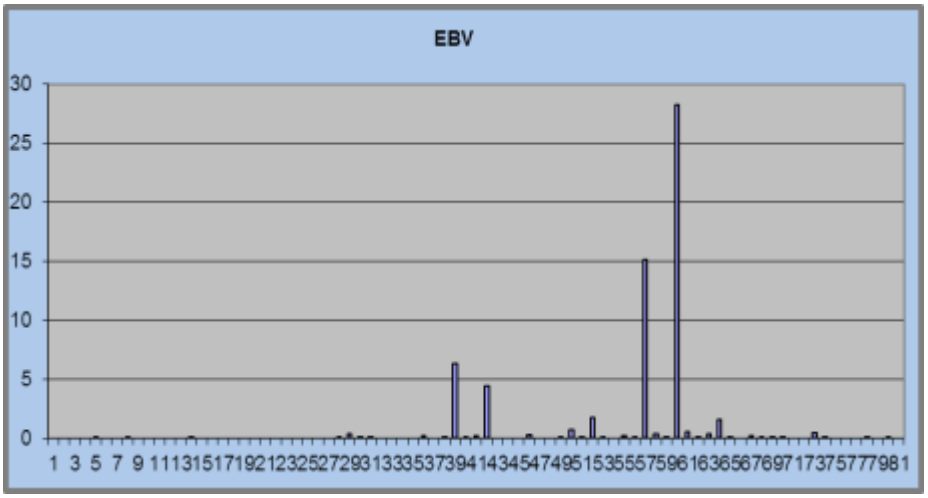

Figure (5): the copy number of EBV in Hodgkin's lymphoma patients

In terms of gender; the majority of patients were male 33 (82.5%) while 7 (17.5%) were females; Figure (6). The results were compatible with the studies by.[13] [14] In which mean of age were 58 and 55 years respectively and the male more common than female, they noticed the males consist 68% and 70% respectively.

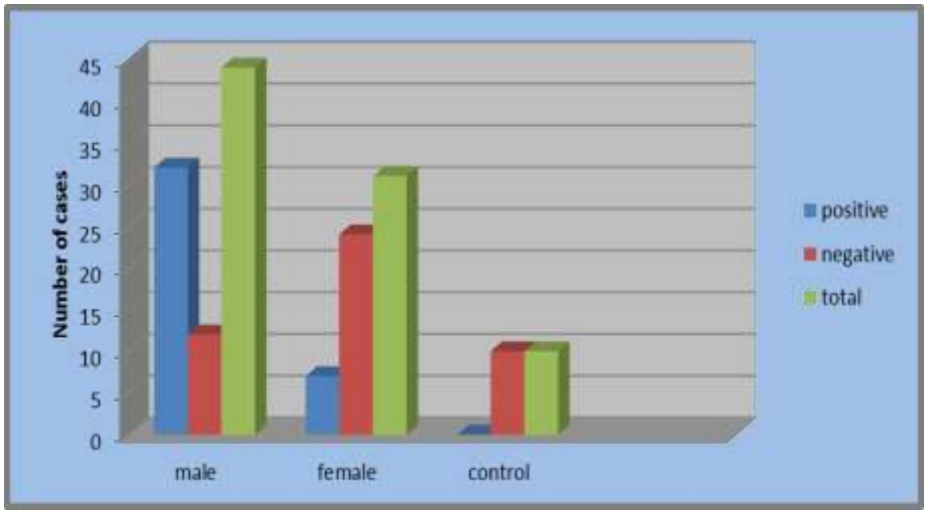

Figure (6): Description of EBV BNRFI according to gander

The distribution of EBV BNRF1 in different subtypes of Hodgkin's lymphoma was as follows: 27(67.5%) of 40 mixed cellularity HD and 13(32.5%) of 26 nodular sclerosing, figure (7). This was significantly higher (P< 0.001) in comparison with the incidence in the control group (0 of 10 individuals; 0%).

Our finding were similar to results reported by[15] who reported that 20 cases positive EBV from 30 total patients of HD were belong to the mixed cellularity subtype HD. Also the results agreement with the results of studies were carried out in other country, in Jordan that EBV was seen in (65%) of mixed cellularity subtype[16] also the results of[17] study occurred in United Arab Emirates noticed that EBV was seen common in mixed cellularity subtype, moreover the study reported by[18] in Egypt showed that mixed cellularity subtype having EBV positive results form about (100%).

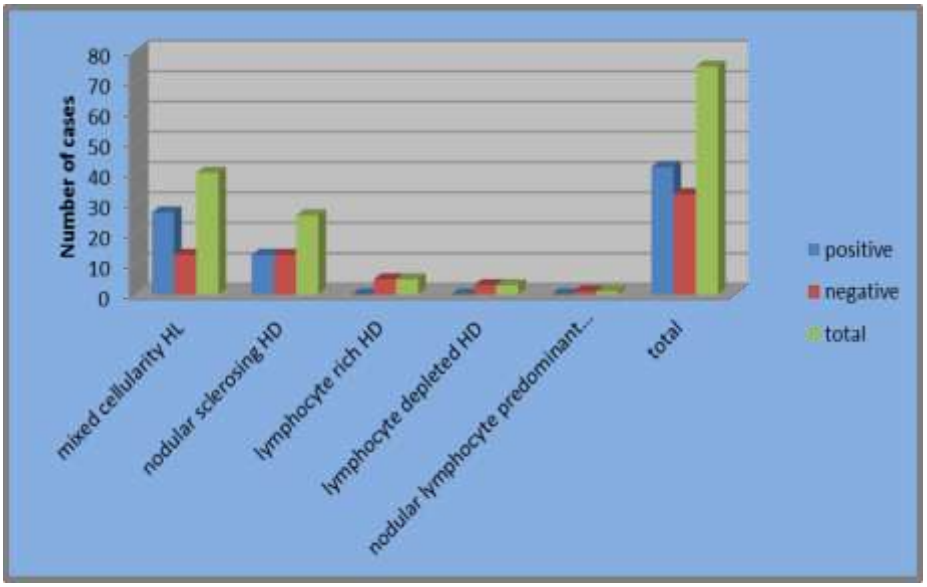

Figure (7): Description of EBV BNRF1 in different Hodgkin's disease

The results of our study reported a relative correlation between EBV DNA levels and Hodgkin's disease in Iraqi patients. These results were in agreement with[19][20] who concluded that EBV DNA have excellent sensitivity and could be used as a biomarker for EBV associated HL when they reported the presence of detectable viral load in 50% of EBV positive HL patients.

The disagreement might be due to the difference in the type of samples that selected for the individual studies, if the patients were taken therapy or no and different of geography.[21]

The EBV positive among HL varies greatly around the world especially with respect to geography, in North America and western Europe, the rate of EBV was detected in 30-50 % of HL patients, while in some parts of Latin America, Africa and Asia the ratio is more higher may be reaching 100% while in Peru and Mexico incidence of EBV positivity among HL ranged from 50-95% and in china was 65%[22] Globally from more studies reported that EBV positive HL account for up 40% of all HL cases, and they have been shown to vary substantially by patient demographic and tumors features, we conclusion the presence of EBV in HL is strongly associated with specific epidemiological features including male gander, mixed cellularity subtype, older adults, low socio-economic status.[23]

In spite of our evolving understanding of the role of EBV in the pathogenesis of disease, but the optimal administration of EBV associated with tumor still indefinite.[24] [25]

Epstein Barr virus is a very widespread virus that is already infecting large number of people around worldwide and capable of persisting for the lifetime in the host.[26] Property that characterizes the herpsviruse is their ability to maintain a latent infection with the virus genome held in the host cells without production of infectious virions, EBV latent gene function is correlate between the factors contributing to the establishment of maintain infection in memory B-cell pool and with the role of the virus in the oncogenic process.[27] Epestian Barr virus targets B lymphocytes via the CD21 receptor and consolidates a latent infection both in vivo and in vitro.[28]

The Hodgkin's lymphoma like other cancers occur because somatic mutations in the genetic material, certain mutations produce cancer by activating oncogenes and inhibit or deactivating tumor suppressor genes that leads to impede the regulation of cell death, differentiation or division, the mutations may be happen spontaneously or occur because the exposure to the radiation, infection by pathogen microorganisms (virus) or carcinogenic substances and may be influenced by genetic factors , all these of agents may develop tumors or cancer.[29] EBV can be considered as the prototype of oncogenic viruses that play as direct transforming agents.[21]

The entity oncogenic herpesvirus (EBV) has been suggested as the major cause for a pathogenetic role due to at least three evidence: the biological mechanism of EBV- mediated B cell transformation, the presence of clonal EBV genomes within HL tumor cells and the three-fold elevated risk of HL in people with a history of infectious mononucleosis.[30]

CONCLUSION

The QC- PCR assay allows accurate quantification of EBV load and show as a tool to assist in diagnosis and management of EBV related lymphoma patient and cancer related with EBV DNA that may be considered as a tumor biomarker.

Despite improvements in our understanding of the pathogenesis of Hodgkin's disease but the precise contribution of EBV remains largely unknown.

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